A STUDY OF THE TERMINAL INNERVATION OF A FAST-ACTING FISH MUSCLE

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This study is concerned with the investigation of the morphology and distribution of the endplates in the intrinsic striated muscle in the wall of the swimbladder of the toadfish, *Opsanus tau*. Recently Skoglund (1959) showed that swimbladder muscle reaches its contraction peak within 5–8 msec. and relaxation is completed in an additional 5–7 msec. Fawcett and Revel (1961) observed by electron microscope studies of the muscle fiber the unique shape and radical disposition of the myofibrils, as well as an elaborate branched sarcoplasmic reticulum. They reported this reticulum has large transverse triads which might indicate an efficient system for intracellular impulse conduction. Because of its unusual morphological and physiological properties, it was thought an understanding of the terminal innervation of this muscle might be of value to future investigators.

Materials and Methods

The experiments were performed on the common toadfish, *Opsanus tau*. Their ages, approximated from the length and sex of the fish (Schwartz, 1963), ranged from less than one to almost eleven years. Animals of both sexes were used. In all experiments the animal was immobilized with a 0.01% (W/V) solution of MS-222 Sandoz in sea water.

Methylene blue perfusion, gold chloride toning and cholinesterase localization were selected to stain the peripheral nerves and their endings.

Intravital staining was accomplished by a methylene blue perfusion technique. The staining fluid was prepared a few hours before use and contained: Methylene blue (Allied Chemical C.I. 52015, Cert. No. 400) 0.25 g., Resorcin (Merck) 0.15 g., NaCl 8.00 g., phosphate buffer (pH 5.4–6.2) 10.00 ml., distilled water 1000.00 ml.

Oxygen was bubbled through the stain solution throughout the procedure. A three-way valve enabled the stain to be drawn into a 50-ml. syringe and then forced through the tubing cannulate while maintaining airtight connections. A midventral incision was made the length of the fish and both the heart and swimbladder were exposed. Extreme care was taken to avoid severing large blood vessels. Two small slits were made in the conus arteriosus, one for the insertion of the cannula and the other for exsanguination. Once inserted the stain was forced through the cannula. When air bubbles were noted the cannula was removed, cleared of the obstruction and then reinserted. A volume of about 150 ml. was injected in 10–15 minutes and no longer than 20 minutes.

Following perfusion small pieces of muscle were removed and placed on loosely piled gauze, allowing full exposure to the air. The tissue was then saturated with staining fluid and after about 20 minutes it acquired a deep blue shade. The

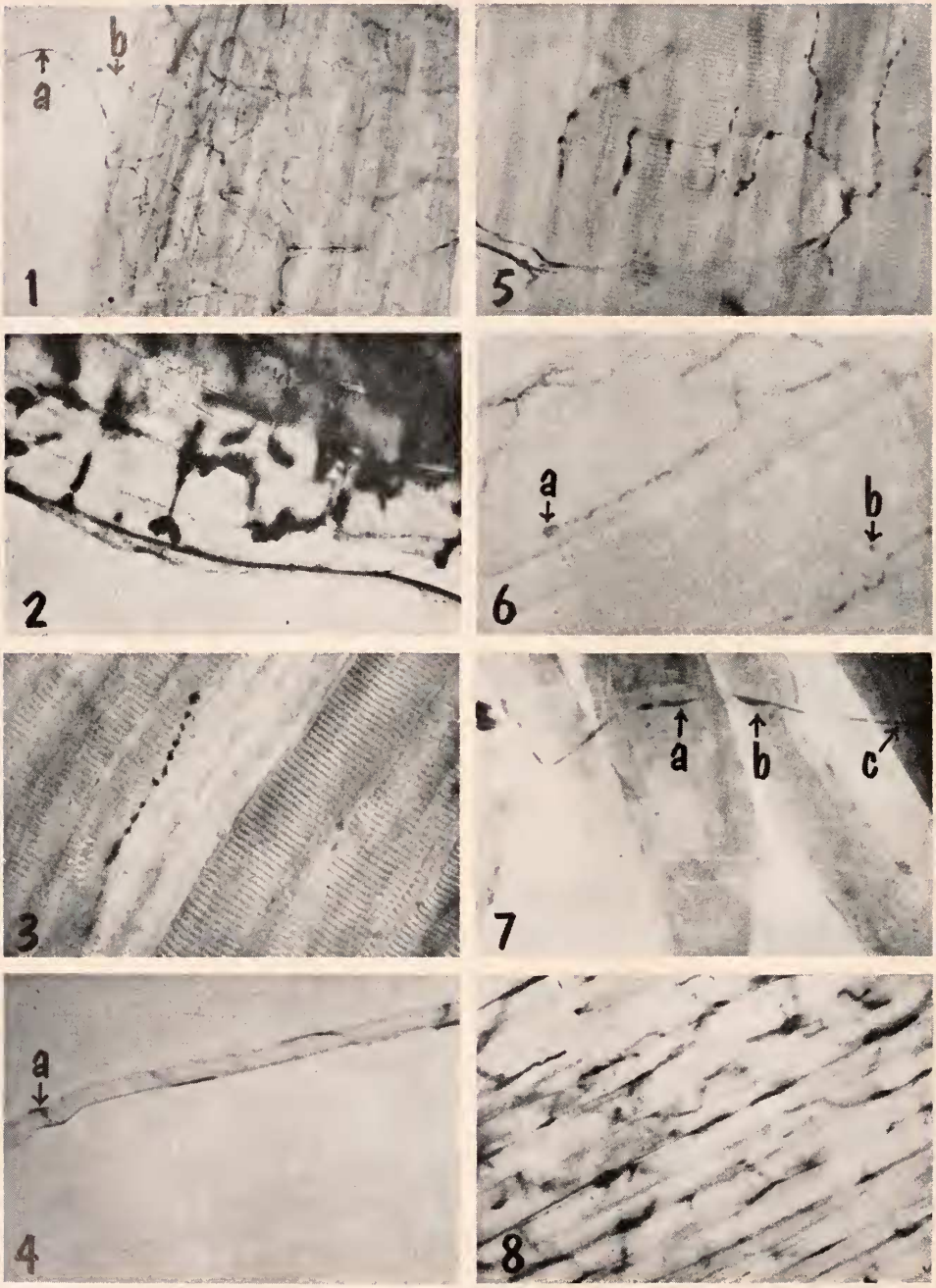

FIGURE 1. This and succeeding figures are of teased toadfish swimbladder muscle. Intramuscular nerve plexus showing terminal innervation. (a) Axionic swelling on preterminal nerve fiber. (b) Single axonic collateral branch with verrucose ending. Methylene blue. × 300.

stained tissue was immediately fixed in chilled 8% ammonium molybdate in 0.8% saline, refrigerated (0–3° C.) overnight, and subsequently washed in several changes of tap water, for a total of two hours.

Dehydration of the tissue was initiated in 95% ethanol for 15 minutes, followed by changes of absolute ethanol and xylene for 15 minutes each. After teasing the pieces into their component fascicles, they were mounted in Permount.

The gold chloride technique used was Cole's (1946) modification of Ranvier's original staining method. Following this treatment muscle fascicles were teased and mounted in glycerine for study.

For acetylcholinesterase localization the technique of Koelle (1951) was employed. Teased preparations were incubated 30 minutes to 60 minutes in acetylthiocholine iodide (AThCh), after treatment with 1.5×10^{-5} *M* diisopropylflurophosphate (DFP), for 30 minutes. Following this treatment the tissues were washed, dehydrated and mounted in Permount.

Results

The intrinsic muscle of the swimbladder consists of two muscle bands composed of short fibers running transversely to the long axis of the muscle. The muscle fibers ranged from 30 μ to 50 μ in diameter.

Neural elements were interpreted in swimbladder muscle by integrating the results of the three staining methods employed in this study. The motor nerves innervating the swimbladder muscle are composed of single fascicles enclosed in perineural sheaths. The nerve fibers within the fascicles appear to be myelinated and are of relatively consistent diameter, ranging from 7 μ to 11 μ. The main nerve trunk passed at right angles to the direction of the muscle fibers, while preterminal fibers branched from the main trunks either in small groups or singly (Figs. 1, 2, 5) and in most cases ran parallel to the long axis of the muscle fibers (Figs. 3, 5, 6).

The preterminal fibers may end in a single endplate (Figs. 2, 3, 4, 6) or unbranched collaterals terminating in bead-like dilatations (Figs. 3, 5). Frequently the marginal, longitudinal, preterminal fibers give off collateral branches which cross the muscle fibers at right angles and terminate marginally on the opposite side of the muscle fiber in club-like expansions or verrucose dilatations about 2 μ in diameter (Figs. 1, 6, 8).

The length of the endplates ranged from 2 μ to 100 μ for the most diffuse type of ending and adjacent endings on the same muscle fiber ranged from 20 μ to 80 μ apart.

Figure 2. Motor endplates and terminal nerve twigs stained for AChE localization. × 1000.

Figure 3. Bead-like ending with enlarged terminus. Methylene blue. × 450.

Figure 4. Isolated muscle fiber showing four junctional areas. AChE localization. × 100.

Figure 5. Preterminal nerve fiber showing diffuse terminal innervation. Methylene blue. × 300.

Figure 6. (a) Shows a discrete ending on a muscle fiber. (b) Shows a single axonic collateral branch with verrucose ending. Methylene blue. × 300.

Figure 7. Preterminal nerve fiber with axonic swellings indicated by arrows. Gold chloride. × 1200.

Figure 8. Acetylcholinesterase localization, showing a variety of neural elements. The darker stained elements terminate the lighter stained preterminal fibers. × 300.

Discrete axonic swellings were seen in terminal myelinated axons stained with all three techniques employed (Figs. 1, 7). Gerebtzoff (1959) reported axonic swellings in the lamprey. The axonic swellings in the toadfish ranged from 9 μ to 25 μ in length and were three to five times wider than the nerve fiber on which they were located. Although the structure of the swellings could not be determined with light microscopy, they stained selectively with Koelle's (1951) histochemical method.

The results of the acetylcholinesterase preparations are shown in Figures 2, 4, and 8. The acetylcholinesteratic activity of the terminal nerve pattern is shown in Figure 8.

In all teased preparations the plexiform pattern of innervation was observed to extend throughout the length of the muscle fiber. It was not possible to determine whether multiple nerve terminations were from the same or different nerve fibers.

Discussion

The results of the present study indicate that there is a variety of terminal nerve elements in swimbladder muscle. Generally, small bundles of nerve fibers tend to intertwine into an intricate plexiform pattern from which the individual motor fibers then separate out into small sprays of indiscrete and discrete endings.

A plexiform pattern of the intramuscular nerve bundles has been cited by Tiegs (1953) in striated muscle of the rabbit and monkey, by Hinsey (1934) in the sartorius muscle of the common frog, and by Cole (1955) and Barets (1955) in the lateral profundus muscle of various fishes.

Sensory endings rarely stain for cholinesterase (Coërs and Woolf, 1959), and only after a prolonged incubation of five hours or more can any faint enzyme activity be detected in them (Coupland and Holmes, 1957; Gerebtzoff, 1959). In addition, the "terminaisons en panier," originally described by Giacomini (Hinsey, 1934) as sensory elements in the fish, have been interpreted as a plexus of motor nerves innervating fast-acting fish muscle (Barets, 1955; Barets and Le Toure, 1956). This corresponds to Tieg's (1953) statement that muscle spindles are absent in fish.

The pattern in extremely rapid extraocular muscles is somewhat similar to that of intrafusal muscle fibers. In addition to multiple equatorial endplates, motor nerve fibers appear to give rise to numerous small branches which innervate series of minute endplate-like structures along the length of the muscle fiber (Gerebtzoff, 1959; Kupfer, 1960). Multiple innervation has been reported for other mammalian muscle fibers (Feindel *et al.*, 1952; Hunt and Kuffler, 1954; Rossi and Cortesina, 1965; Mahran and Sakla, 1965) and occurs in many avian muscles (Ginsborg, 1960; Ginsborg and Mackay, 1961).

Hoyle (1957) examined the twitch muscle fibers of the locust, which structurally resemble the muscle fibers in toadfish swimbladder muscle. He states that multiple innervation might permit impulses to reach the termination at several points along the muscle fiber in less time than permitted by a single myoneural junction. Wiersma (1957) states that in arthopods the nerve fiber itself is often responsible for the conduction of the impulse along the fiber rather than the propagated depolarization of the sarcolemma.

According to Hess (1962, 1963), multiple endings along a single fiber may vary somewhat in form but never to the extent that one fiber may have classically-defined "en grappe" and "en plaque" forms. Garvin (1925) refrained from classifying the diverse endplates in the panniculus carnosus of the hedgehog as either "en grappe" or "en plaque." Wilkinson (1929) saw a similar variation of the endings occurring on different muscle fibers in several species of lizards and frogs. He found different forms terminating the branches of the same motor neuron and associated diffuse endings with smaller fibers and plate-like endings with muscle fibers of larger diameter. He suggested that the bead-like endings of the terminal axons represented growing ends of nerve and that the grape-like endings are a developmental form, both ontogenetically and phylogenetically. More recently, Csillik (1961) proposed that the diffuse ending is a regenerative as well as ontogenetically immature form of the plate-like ending.

Because of the variety of nerve terminals observed in the swimbladder muscle, it was difficult to classify them specifically. Studies are in progress at the present time to attempt to solve this discrepancy.

Summary

1. Histochemical localization of the enzyme, acetylcholinesterase (AChE), methylene blue perfusion, and gold chloride were employed in studying the motor nerve innervation of the intrinsic striated muscle located in the wall of the swimbladder of the toadfish, *Opsanus tau*.

2. A plexiform pattern of multiple innervation was revealed.

3. Both preterminal nerve fibers and nerve terminals showed acetylcholinesterase activity.

4. A wide variation of shape, size and distribution of nerve endings was seen. The endplates ranged from 2 μ to 70 μ in length and were spaced from 20 μ to 480 μ along a muscle fiber.

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